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10. Thank you!

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A Molecular Switch for the Consolidation of Long-Term Memory: cAMP-Inducible Gene Expression

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As this symposium illustrates, molecular genetics has brought about a dramatic unification within the biological sciences. The ability to sequence genes, and to infer the amino acid sequence of the proteins they encode, has revealed unanticipated relationships between proteins encountered in different contexts. As a result, there is now a general blueprint for cell function that provides a common conceptual framework for several, previously unrelated, disciplines: genetics, biochemistry, immunology, development, cell biology, and neurobiology. A parallel and potentially equally profound unification is occurring between neural science, the science of the brain, and cognitive psychology, the science of the mind. The ability to study the neuronal basis of mental function is providing a new impetus for examining cognitive processes such as perception, action, language, learning, and memory. To what degree can these two independent and disparate strands be brought together? Can molecular biology enlighten the study of mental processes? In this brief review we outline the possibility of a ***molecular biology of cognition***, using as examples several simple forms of memory and learning in invertebrates and vertebrates.

LEARNING IS NOT A UNITARY MENTAL FACULTY BUT HAS AT LEAST TWO MAJOR FORMS

One of the major conceptual advances of recent cognitive psychological studies is the finding that learning is not a unitary faculty of the mind, but

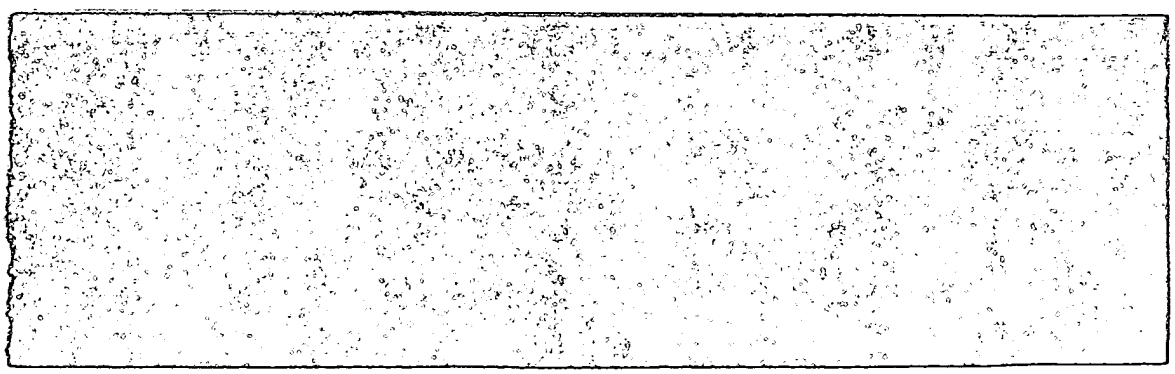
consists of at least two distinct mental processes (for review, see Ref. 1): learning about people, places, and things (explicit or declarative forms of learning), and learning motor skills and perceptual strategies (implicit or procedural forms of learning). These two major forms of learning have been localized to different neural systems within the brain.[2,3] Explicit learning importantly involves regions within the temporal lobe of the cerebral cortex, including the *hippocampus*, whereas implicit learning involves only the specific sensory and motor systems recruited for the particular perceptual or motor skills utilized during the learning process.[4] As a result, implicit learning can be studied in a variety of reflex systems, including those of invertebrates such as *Aplysia*, *Limax*, *Hermissenda*, and *Drosophila*.[5,6] By contrast, explicit forms of learning are best studied in mammals.[7-9]

The finding of two phenotypically different forms of learning raises the question: To what degree do they share common molecular steps? One clue to shared mechanisms comes from the study of memory storage, that is, the retention of information acquired through learning. Memory for both implicit and explicit forms of learning has stages, and is commonly divided into at least two temporally distinct components: short-term memory that lasts minutes to hours, and long-term memory that can last days, weeks, or even years. Studies of memory storage for both implicit as well as explicit learning indicate that both involve a switch or *consolidation mechanism* that appears to have common features. In both cases the consolidation of memory from a labile short-term form to a stable, self-maintained long-term form requires the induction of genes and proteins. With both implicit and explicit memory a transient application of inhibitors of mRNA and protein synthesis selectively blocks the induction of long-term memory without affecting short-term memory.[10-13] A similar application of inhibitor has no effect on the maintenance of long-term memory once it is established.

What genes and proteins contribute to the consolidation switch that turns on the long-term process? To what degree are they conserved in the two major forms of learning? Here, we first describe the insights that have been gained from studies of elementary forms of implicit learning in *Aplysia* and *Drosophila*. We then briefly consider long-term potentiation (LTP) in the hippocampus, a type of synaptic plasticity that is thought to contribute to explicit forms of memory storage in mammals.

SENSITIZATION OF THE GILL-WITHDRAWAL REFLEX IN *APLYSIA*: AN IMPLICIT FORM OF LEARNING

The molecular mechanisms contributing to implicit memory storage have been most extensively studied for the gill-withdrawal reflex of the marine



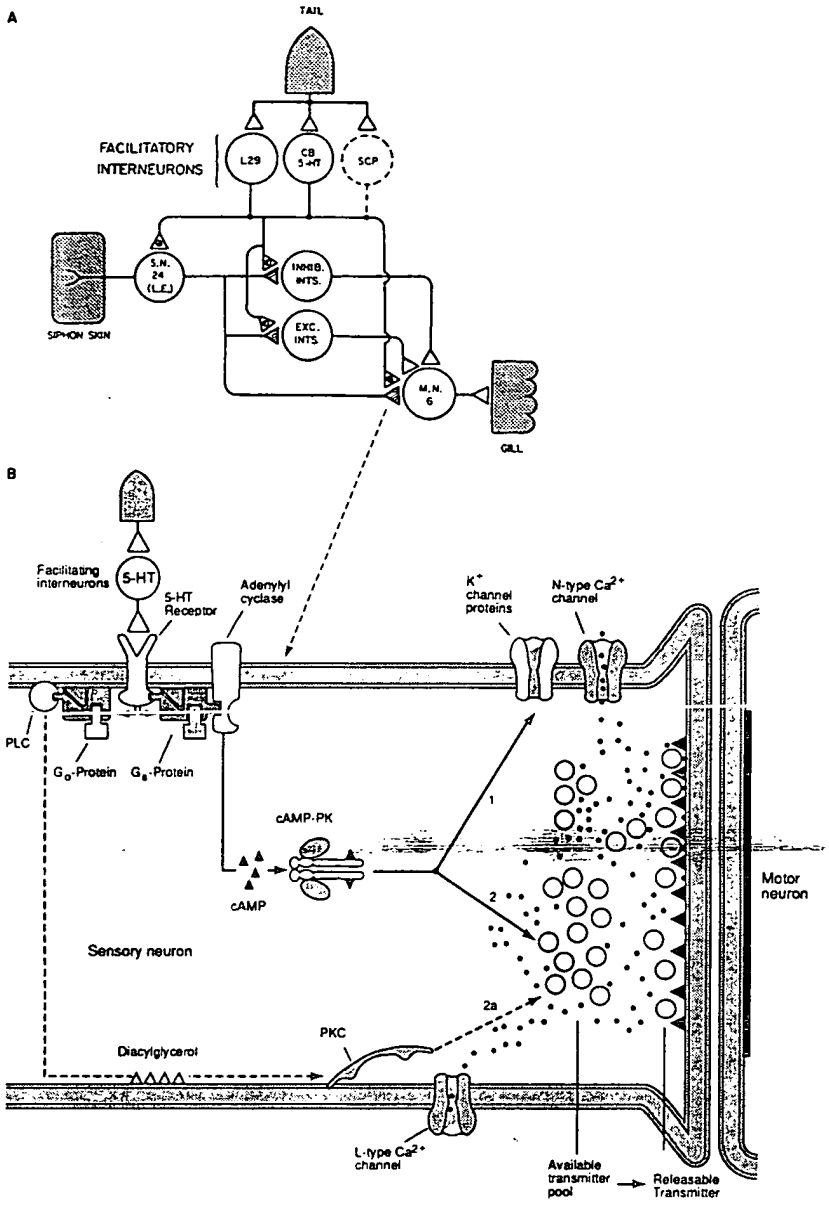
snail, *Aplysia californica*. As is true for other defensive reflexes, the gill-withdrawal reflex can be modified by several different forms of learning. We will focus here on only one: sensitization.

Sensitization is a form of learning in which an animal learns to strengthen its reflex responses to previously neutral stimuli following the presentation of an aversive stimulus. When *Aplysia* is presented with a noxious stimulus to the tail, the animal recognizes the stimulus as aversive and learns to enhance its reflex response to an innocuous stimulus applied to the siphon. The duration of the consequent memory is a function of the number of sensitizing stimuli applied to the tail.[14] A single noxious stimulus to the tail produces short-term memory. The resulting enhancement of the withdrawal reflex lasts for minutes and does not require new protein synthesis.[11,15] By contrast, four or five noxious stimuli to the tail produce long-term memory lasting one to two days, which requires new protein synthesis. Further training leads to an even more enduring memory reflected as a reflex enhancement lasting several weeks.

A number of key components of the neural circuit for the gill-withdrawal reflex have been identified. Sensitization leads to modification of several of these (FIG. 1). One site that has been studied extensively is the monosynaptic connection between the sensory and motor neurons. This connection carries a representation of both short- and long-term memory, which is expressed as an enhanced release of transmitter from the synaptic terminals of the sensory neurons (presynaptic facilitation) (FIG. 1). The molecular steps leading to enhanced transmitter release activated by short-term memory involve a phosphorylation cascade mediated by cyclic AMP and protein kinase A[16,17] as well as protein kinase C.[17-19] Long-term memory leads to enhanced transmitter release by means of cAMP-mediated gene expression.

The monosynaptic component between the sensory and motor neurons of the gill-withdrawal reflex can be reconstituted in dissociated cell culture. This has allowed a more detailed analysis of the mechanisms involved in presynaptic facilitation of transmitter release. The reconstituted circuit in culture undergoes presynaptic facilitation in response to serotonin (5-HT), a neuromodulator released *in vivo* during sensitizing stimulation applied to the tail.[14,15,20-22] As with behavioral sensitization,[11] the amplitude and duration of the synaptic facilitation *in vitro* is a function of the number of applications of 5-HT. A brief pulse of 5-HT or cAMP produces a short-term facilitation lasting only minutes; 4 or 5 pulses of 5-HT or cAMP separated by 20-min intervals elicit a long-term facilitation lasting more than 24 hours. As with behavioral sensitization, long-term facilitation requires RNA and protein synthesis during a brief critical time window that corresponds to the period of 5-HT application.[15] One hour after the last 5-HT application, long-term facilitation is no longer susceptible to disruption by inhibitors of RNA or protein synthesis.[15] This consolidation period evident on the cellular level, during which long-term fa-

cilitation is capable of being blocked by inhibitors of protein and RNA synthesis, corresponds to the consolidation phase of long-term memory. This finding of an elementary cellular representation of consolidation allows us to ask: What is the molecular nature of memory consolidation?



LONG-TERM FACILITATION IS ASSOCIATED WITH THE SYNTHESIS OF "EARLY" AND "LATE" PROTEINS

Since long-term synaptic plasticity, like long-term memory, requires protein synthesis during a consolidation period, Barzilai *et al.*[23] first analyzed the proteins synthesized in *Aplysia* sensory neurons after exposure to sensitizing training. They found that both behavioral training and application of 5-HT or cAMP induce a sequence of early and late changes in protein synthesis, consistent with the consolidation period's being a time during which a cascade of gene expression is activated, allowing early regulatory proteins to control the expression of late effector genes (FIG. 2). The early proteins have the features of immediate-early gene products. Their expression is rapid (within 15 to 30 min), transient, and dependent on transcription. The immediate-early genes encode not only effector proteins, but also regulatory proteins such as transcription factors that act on later effector genes.

cAMP-DEPENDENT GENE EXPRESSION IS REQUIRED FOR LONG-TERM FACILITATION

The data from Barzilai *et al.* suggested that repeated application of facilitatory neurotransmitter activates not only cytoplasmic second messengers, but also a cascade of early and late genes whose products are required for long-term synaptic plasticity.[24] How does cAMP activate transcription? What immediate-early genes are activated by 5-HT and cAMP? What are the late effector genes? Studies by Bernier *et al.*[25] and Bacskai *et al.*[26] showed that serotonin, acting on the sensory neurons, stimulates the synthesis of cAMP, which activates the protein kinase A (PKA) catalytic subunit by binding to the regulatory subunit. By imaging the free catalytic and regulatory subunits

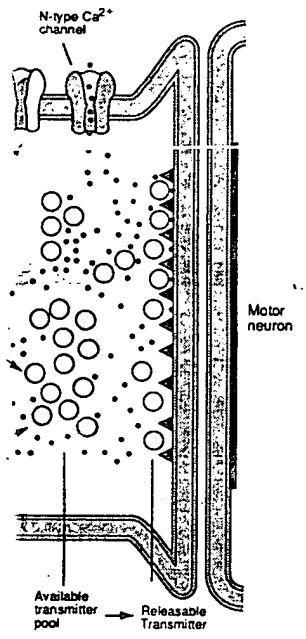
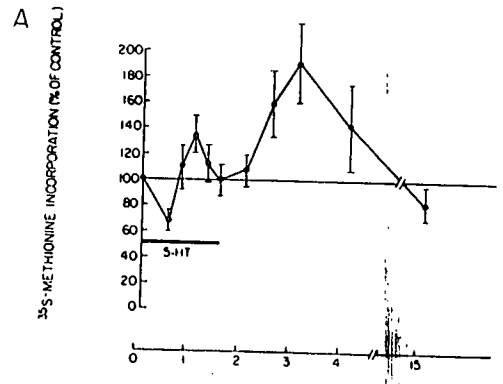


FIGURE 1. Presynaptic facilitation underlies sensitization of the gill-withdrawal reflex in *Aplysia*. (A) Sensitization is produced by a noxious stimulus to the tail of *Aplysia*. Sensory neurons innervating the tail excite facilitating interneurons, some of which use serotonin (5-HT) as their transmitter. Inhibitory and excitatory interneurons make synapses with the axonal terminals of sensory neurons from the siphon skin, where they enhance transmitter release by presynaptic facilitation. (B) Postulated biochemical steps during presynaptic facilitation in the sensory neuron. 5-HT enhances transmitter release by binding to a receptor that engages a G-protein, which increases the activity of adenylyl cyclase, thereby increasing cAMP levels in the presynaptic terminal. cAMP activates the cAMP-dependent protein kinase (PKA) by releasing the enzyme's catalytic subunit from its complex with the regulatory subunit. The catalytic subunit phosphorylates K^+ channels, thereby decreasing the K^+ current, prolonging the action potential, increasing Ca^{2+} influx through N-type Ca^{2+} channels, and augmenting transmitter release. In addition, 5-HT also increases the availability of transmitter by mobilizing vesicles to a releasable pool near the active zones where release occurs (pathway 2). This second pathway involves both PKA and protein kinase C (PKC), which is activated by 5-HT via a G-protein that activates a phospholipase (PLC) that produces diacylglycerol in the membrane. Diacylglycerol then stimulates PKC.



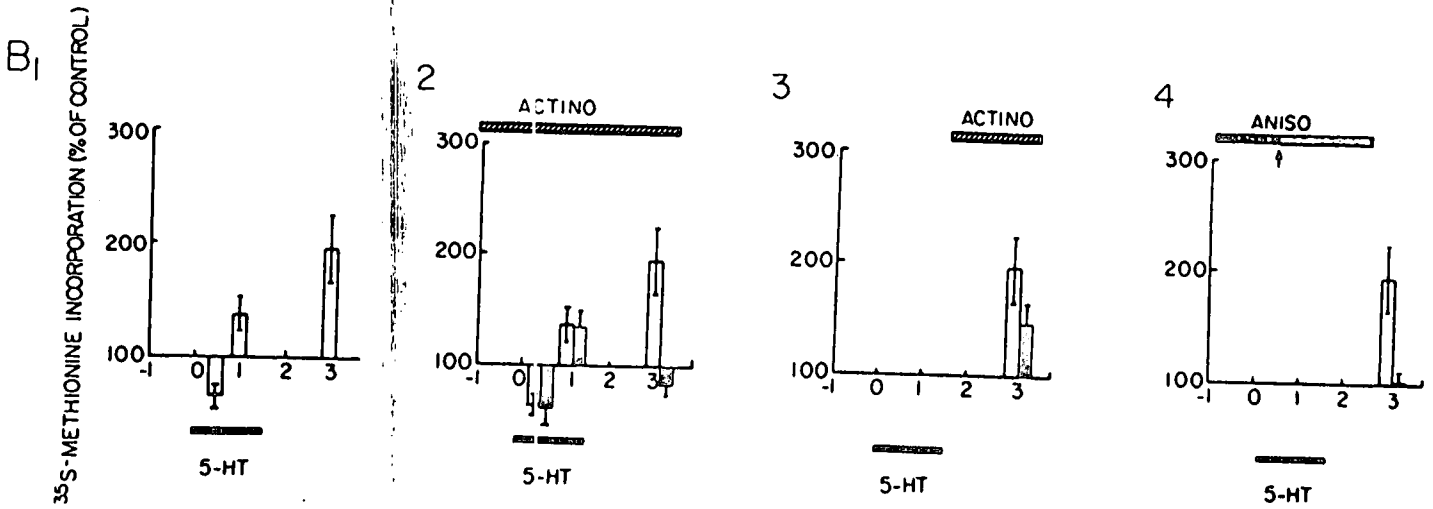
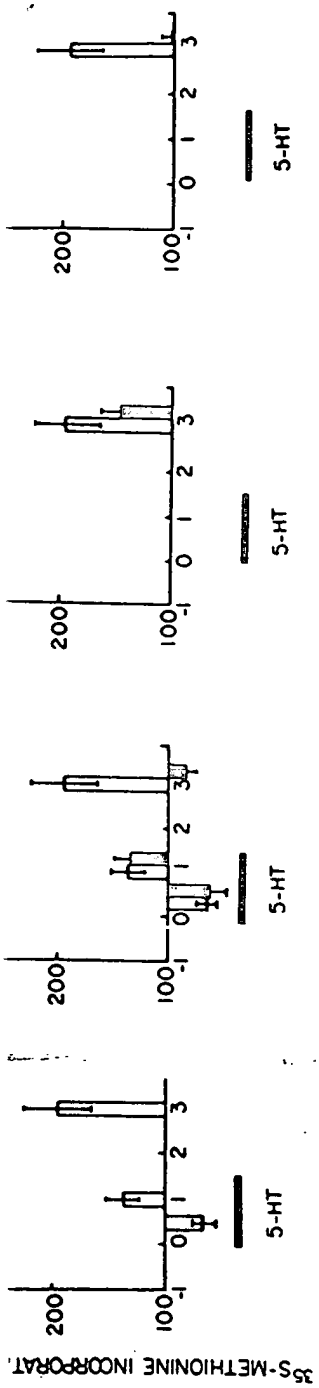


FIGURE 2. The effect of a 1.5-hr application of 5-HT on the rate of overall protein synthesis in *Aplysia* sensory neurons. **(A)** Incorporation of [^{35}S]Met into pleural sensory cluster proteins at different times during and after treatment with 5-HT. The results are expressed as the ratio of [^{35}S]Met incorporated into 5-HT–treated cluster over unstimulated cluster from the same animal. The *bars* represent SEM. **(B)** The effect of actinomycin and anisomycin on the peaks of [^{35}S]Met incorporation induced by 5-HT. **(B_1)** The *open histograms* represent the 0.5-hr trough, 1-hr peak, and 3-hr second peak. The data are the same as those of **(A)**. **(B_2)** To block transcription, the pleural sensory clusters were preincubated with 100 μg/ml actinomycin-D (a concentration that does not inhibit protein synthesis) 1 hour prior to treatment with 5-HT. The *solid histograms* represent the actinomycin-D–treated clusters. **(B_3)** The experimental protocol was identical to that described in **(B_2)**, except that actinomycin-D was applied 90 min after the sensory neurons were treated with 5-HT. **(B_4)** To transiently block translation, the sensory cells were preincubated with 20 μM anisomycin beginning 60 min before and lasting until 30 min after the application of 5-HT.

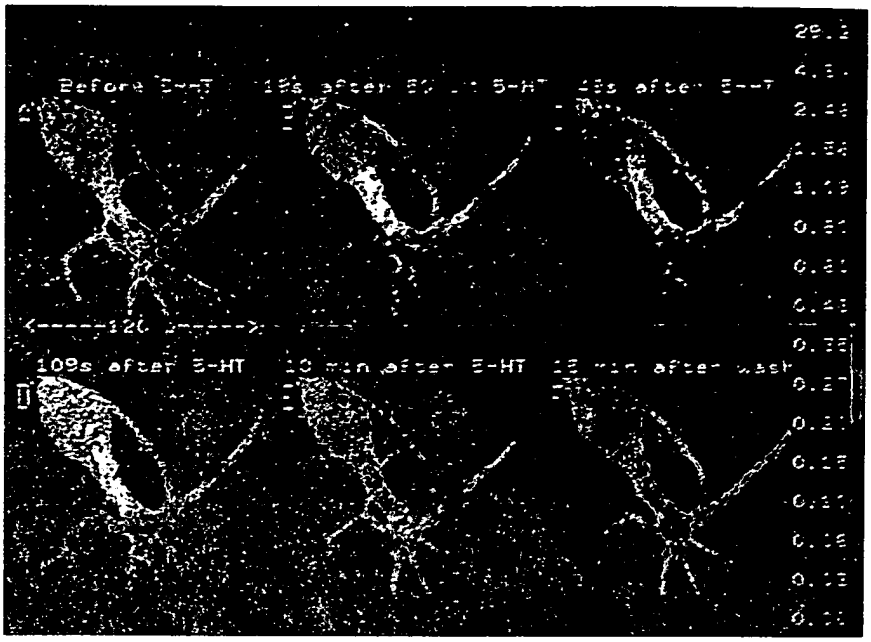


of PKA, Bacskai *et al.* found that a single pulse of 5-HT increases the free catalytic subunit concentration in the cytoplasm of the sensory neuron and especially in the presynaptic terminals (FIG. 3A).[26] With repeated pulses of 5-HT, the catalytic subunit translocates to the nucleus of the sensory neurons (FIG. 3A),[26] where it appears to phosphorylate one or more CREB-related transcription factors that activate cAMP-inducible genes.[27,28] In fact, Dash *et al.* and Kaang *et al.* found data suggesting that one of the substrates of protein kinase A is a CREB-like protein that binds to the cyclic AMP response element (CRE) (FIGS. 3B and 3C). By injecting oligonucleotides containing somatostatin CRE into sensory neurons, Dash *et al.* blocked long-term facilitation without affecting short-term facilitation (FIG. 3B), showing that CRE-binding proteins are critical for expression of long-term facilitation. Kaang *et al.*[28] expressed in the sensory neurons of *Aplysia* a chimeric transacting factor consisting of the mammalian CREB activation domain fused to a GAL4 DNA-binding domain. This fusion protein was able to transactivate the reporter gene (chloramphenicol acetyltransferase) in response to repeated 5-HT application (FIG. 3C). They then tested whether endogenous phosphorylation by PKA was required for the induction of this transacting activity. They compared the activity of the wild-type chimera CREB-GAL4 to a mutant chimera (CREB-GAL4 SA 119) in which the residue serine 119 (essential for the activity of mammalian CREB), was substituted with an alanine, and found that this substitution abolished the ability of 5-HT to induce the transacting activity of CREB-GAL4. In addition, they found that the kinase essential for this activity is likely PKA, since a mutation that inactivates only the PKA phosphorylation site (the substitution of arginine 117 with an alanine) but leaves the CaM kinase consensus site intact blocked the 5-HT-dependent transactivation (FIG. 3C). Taken together, these data imply that CREB-related transcription factors are required for long-term facilitation and are activated by the PKA-dependent phosphorylation induced by 5-HT.

ApC/EBP IS AN IMMEDIATE-EARLY GENE INDUCED DURING THE CONSOLIDATION PHASE OF LONG-TERM FACILITATION IN *APLYSIA*

To understand which cAMP-dependent transcriptional events are activated during long-term facilitation, we next focused on cAMP-regulated transcription factors. Some of the transcription factors known to be activated by cAMP belong to a family known as CCAAT enhancer-binding protein (C/EBP). A member of this family, C/EBPβ, is expressed in the rat pheochromocytoma PC12 cell line, where it has been shown to be activated by cAMP and to regulate the expression of the *c-fos* gene by binding to the sequence ATTAGGACAT (enhancer response element, ERE) in the *c-fos* promoter.[29] Since nerve cells

A1


Before 5-HT
109s after 5-HT
10 min after 5-HT
12 min after wash

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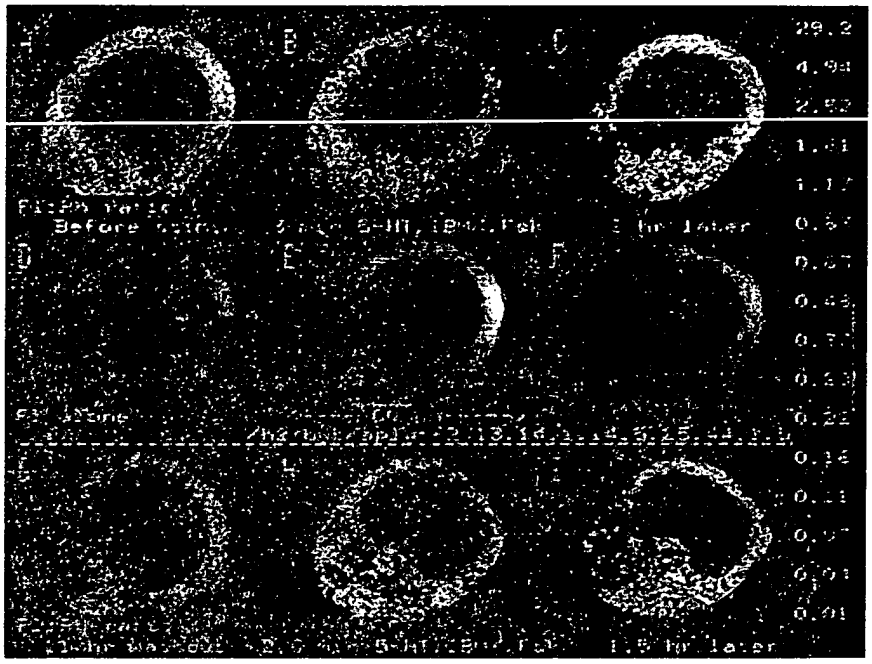
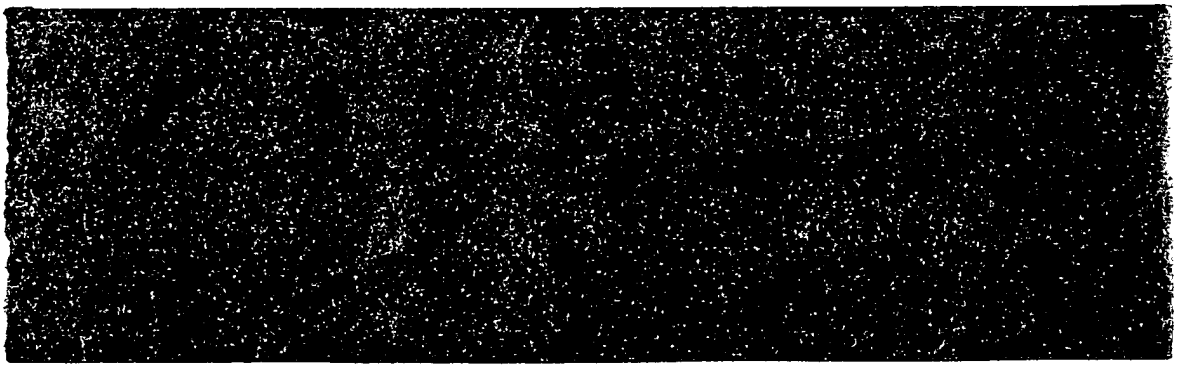

Before 5-HT
1 hr later



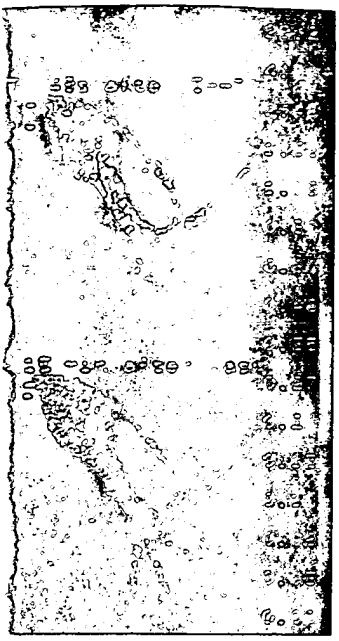


FIGURE 3A1. Gradient in [cAMP] in *Aplysia* sensory neurons after uniform bath application of serotonin (5-HT). A single, cultured *Aplysia* sensory neuron was microinjected with FICRhR. Digital fluorescence images are the result of simultaneous acquisition of two confocal single-wavelength emission images (500 to 530 nm and < 560 nm) at a plane just above the glass substream. After subtraction of background, a ratio was calculated (short over long wavelength), and the image was pseudocolored from blues to reds, which correspond to low to high ratios and low to high concentrations of free cAMP (scale on *right* in μM cAMP). (**A**) Before treatment. (**B**) After uniform bath application of 50 μM 5-HT, a striking gradient of [cAMP] develops between the cell body and the distal processes. The increase in [cAMP] persisted with some habituation while 5-HT remained in the bath (**C** through **E**), but after 5-HT was washed away (**F**), [cAMP] returned to near control levels (**A**).

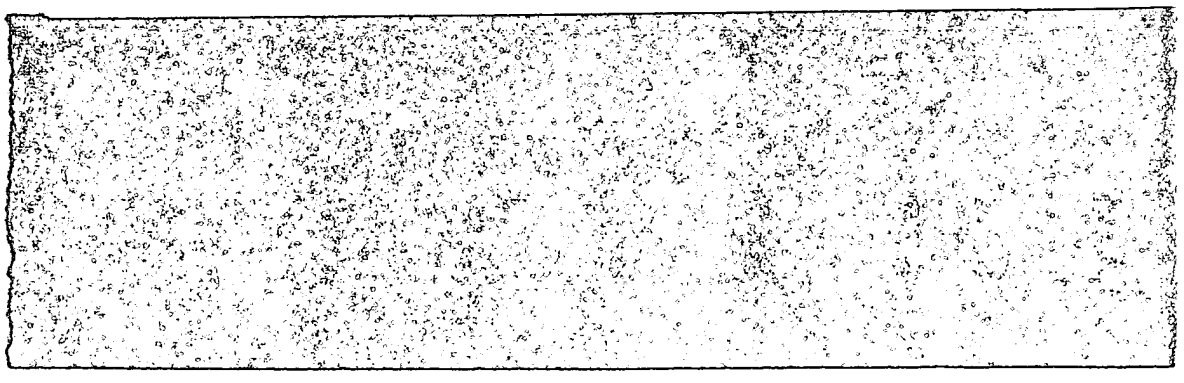
FIGURE 3A2. Translocation of C subunit of cAMP-dependent protein kinase into and out of the nucleus. A single neuron was microinjected with FICRhR and imaged at a plane 20 to 30 μm above the cover slip (**A**). The nucleus excluded the labeled protein and was therefore not fluorescent; the cytoplasm, however, was very bright and exhibited negligible concentrations of cAMP. (**B**) Soon after application of 25 μM forskolin, 0.1 mM isobutylmethylxanthine (IBMX), and 20 μM 5-HT to raise intracellular [cAMP], the fluorescence ratio increased in the cytoplasm, although the nucleus remained dim. (**C**) The nucleus became brightly fluorescent 2 hours later. This apparent high [cAMP] in the nucleus is an artifact generated by entry of C subunits and exclusion of R subunits, causing a fluorescein/rhodamine ratio higher than that caused by dissociated holoenzyme. (**D** through **F**) Single-wavelength images of fluorescein fluorescence (distribution of C subunit) from the corresponding images of (**A**) through (**C**). Confocal sections at different planes of focus (not shown) confirmed that the C subunit entered the nucleus and was not just concentrated around its periphery. The R subunits stayed mostly or completely within the cytoplasm, as determined by excitation of rhodamine alone (not shown) or by red pseudocolor of nucleus in C. (**G**) The same cell was washed and allowed to recover overnight. The emission in the cytoplasm fell to low levels, indicating reconstitution of holoenzyme, and the nucleus was again relatively devoid of fluorescence. Blue patches probably represent degradation of protein still labeled with rhodamine. (**H**) After treatment with 25 μM forskolin and 0.5 mM IBMX, the fluorescence ratio increased, showing that, after more than 12 hours in a cell, much of the FICRhR was still responsive to changes in [cAMP]. (**I**) Nuclear translocation could be observed again after an additional 1.5 hours of stimulation.

of *Aplysia* contain specific binding activity toward ERE, Alberini *et al.*[30] used the ERE to isolate a clone that displayed specific binding activity toward C/EBP DNA binding elements. The protein sequence inferred from the DNA sequence corresponds to a 286–amino acid polypeptide with the characteristic basic region-leucine zipper (b-zip) domain at the C-terminus that is highly homologous to the b-zip domains of the C/EBP family members. The sequence of the genomic ApC/EBP showed that in the 5′ region of the gene a nonpalindromic CRE site is present (ApCRE) 19 bp upstream from the putative TATA box. This site may represent a regulatory element recognized by CREB-like DNA binding proteins.

ApC/EBP IS REQUIRED FOR LONG-TERM FACILITATION

Where is ApC/EBP expressed? How is it regulated? Does it have a role in learning-related synaptic facilitation? To address these questions, we first investigated the expression of ApC/EBP by determining its mRNA concentration in untreated or 5-HT-treated animals. The level of ApC/EBP expression was undetectable in untreated CNS, but it increased significantly after a 2-hr exposure to 5-HT, 8-Bromo-cAMP, or forskolin (FIG. 4A).

The induction of mRNA ApC/EBP by 5-HT is rapid. It is detectable within 15 minutes after exposure to either treatment (FIG. 4B). To determine whether this rapid induction is caused by the direct action of a constitutively expressed factor, we examined the action of 5-HT on ApC/EBP mRNA in the presence of protein synthesis inhibitors (anisomycin or emetine). In the presence of a protein synthesis inhibitor, 5-HT caused a superinduction of the ApC/EBP mRNA (FIG. 4A), indicating that ApC/EBP transcription is induced by 5-HT and cAMP as an immediate-early gene via a constitutively expressed transcription factor. We therefore next investigated the effects of blocking the activity of ApC/EBP on short- and long-term facilitation of sensory-motor synapses in reconstituted monosynaptic circuits using three different approaches. First, we interfered with the binding of the transcription factor to its DNA binding element by injecting ERE oligonucleotides that compete for the binding activity of the endogenous ApC/EBP to its target sequence in the nucleus of the sensory cells. Second, we blocked specifically the synthesis of endogenous ApC/EBP by injecting ApC/EBP antisense RNA into the sensory cells. Third, we blocked the binding activity of ApC/EBP to its DNA target sites by injecting into the sensory cells a specific antibody able to disrupt the binding of ApC/EBP and its target sequences. We found that all of these conditions blocked long-term facilitation (FIG. 5). By contrast, the injection of the same molecules had no effect on short-term facilitation (FIGS. 5A3, B3, C3). These data show that the binding of ApC/EBP to its target DNA sequences is required in order to induce long-term facilitation lasting 24 hours.



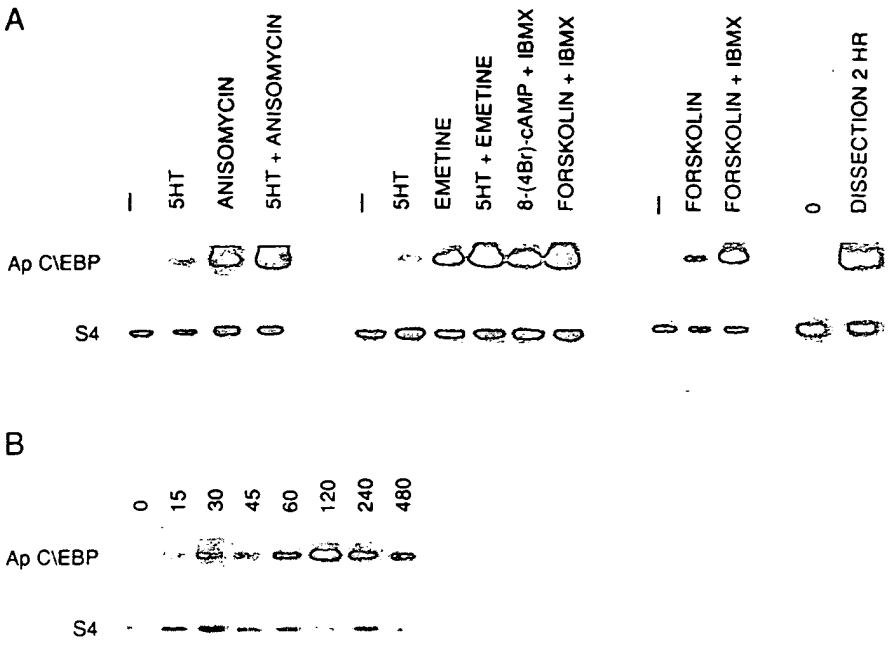


FIGURE 4. Induction of ApC/EBP mRNA. **(A)** ApC/EBP mRNA expression in CNS of untreated *Aplysia*, of *Aplysia* treated *in vivo* with the indicated drugs for 2 hr at 18°C, or dissected without treatment and kept at 18°C in culture medium. Four independent experiments are shown, in which 10 μg of total RNA extracted from CNSs of untreated (–) or treated *Aplysia*, as indicated, were electrophoresed, blotted, and hybridized with ^{32}P-labeled ApC/EBP (*top*) or S4 (*bottom*) probes. The latter encodes the *Aplysia* homologue of S4 ribosomal protein,[59] which is constitutively expressed and used as a loading control. 0 indicates RNA extracted immediately after dissection of *Aplysia* CNS. Two-hour dissection represents RNA extracted from *Aplysia* CNS dissected and incubated in culture medium for 2 hr at 18°C. **(B)** Time course of ApC/EBP mRNA induction following 5-HT treatment. Times of treatment are indicated. Five μg of total RNA extracted from total CNS of *in vivo* treated *Aplysia* were analyzed as described in **(B)**.

How long does the transcription factor need to be active? One possibility is that the binding of ApC/EBP to its target sequences is required throughout the maintenance period of the facilitation. Alternatively, the long-term facilitation may become self-perpetuating as a result of subsequent expression of stable, effector genes. To distinguish between these hypotheses, we injected ERE oligonucleotides into sensory cells at various times from 1 to 12 hours after giving five pulses of 5-HT. We found that the blocking effect of the specific oligonucleotide was progressively reduced when the injection was performed at longer intervals after the training, with facilitation no longer affected by the injection at 12 hours after the training (FIG. 6). Therefore, the induction of ApC/EBP during the 5-HT treatment leads to the activation of

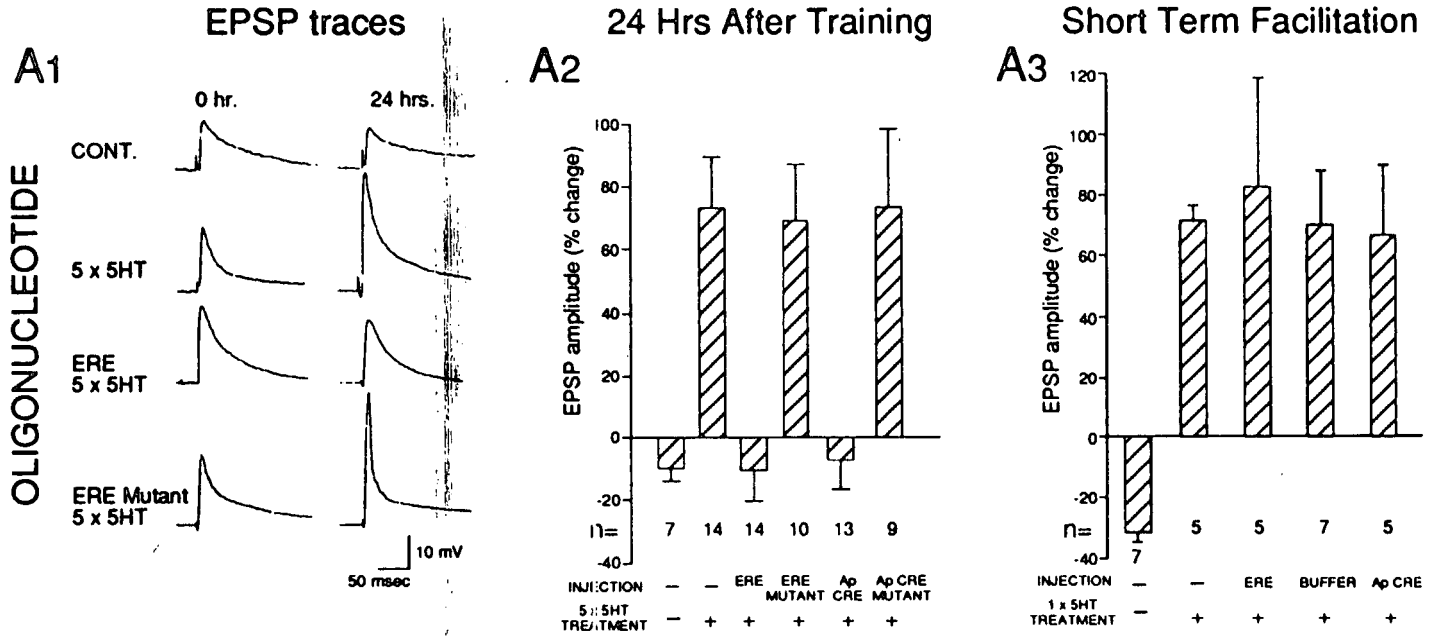


FIGURE 5A. Injection of ERE oligonucleotides blocks 5-HT–induced long- but not short-term facilitation in sensory motor synapses. **(A1)** Examples of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr) and 24 hr after 5-HT treatment. Injection of the ERE oligonucleotide, but not of the corresponding mutant (ERE Mutant) blocks the 5-HT–induced increase in EPSP amplitude at 24 hr. The control culture did not receive 5-HT applications or oligonucleotide injections. **(A2)** *Bar graph* representing the effects of oligonucleotide injections in long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after 5-HT treatment. A one-way analysis of variance and Newman Keuls' multiple-range test indicate that 5-HT treatment significantly increases the EPSP amplitude in noninjected cells, as well as in ERE Mutant or ApCRE Mutant injected cells, relative to the control (not 5-HT–treated and noninjected cells) ($p < 0.01$). On the contrary, the EPSP amplitude change in ERE or ApCRE injected cells was not significantly different from that of control cells that were neither injected nor treated. **(A3)** Bar graph representing the mean EPSP amplitude percentage change ± SEM of short-term facilitated cells injected with ERE oligonucleotides or with ApCRE, or with buffer. A one-way analysis of variance and a comparison of the means show a significant effect of 5-HT in increasing EPSP amplitude in noninjected, in ERE, in ApCRE, or in buffer-injected cultures compared to the control ($p < 0.01$).

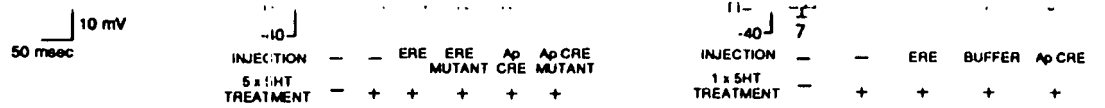


FIGURE 5A. Injection of ERE oligonucleotides blocks 5-HT–induced long- but not short-term facilitation in sensory motor synapses. **(A1)** Examples of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr) and 24 hr after 5-HT treatment. Injection of the ERE oligonucleotide, but not of the corresponding mutant (ERE Mutant) blocks the 5-HT–induced increase in EPSP amplitude at 24 hr. The control culture did not receive 5-HT applications or oligonucleotide injections. **(A2)** *Bar graph* representing the effects of oligonucleotide injections in long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after 5-HT treatment. A one-way analysis of variance and Newman Keuls' multiple-range test indicate that 5-HT treatment significantly increases the EPSP amplitude in noninjected cells, as well as in ERE Mutant or ApCRE Mutant injected cells, relative to the control (not 5-HT–treated and noninjected cells) ($p < 0.01$). On the contrary, the EPSP amplitude change in ERE or ApCRE injected cells was not significantly different from that of control cells that were neither injected nor treated. **(A3)** Bar graph representing the mean EPSP amplitude percentage change ± SEM of short-term facilitated cells injected with ERE oligonucleotides or with ApCRE, or with buffer. A one-way analysis of variance and a comparison of the means show a significant effect of 5-HT in increasing EPSP amplitude in noninjected, in ERE, in ApCRE, or in buffer-injected cultures compared to the control ($p < 0.01$).

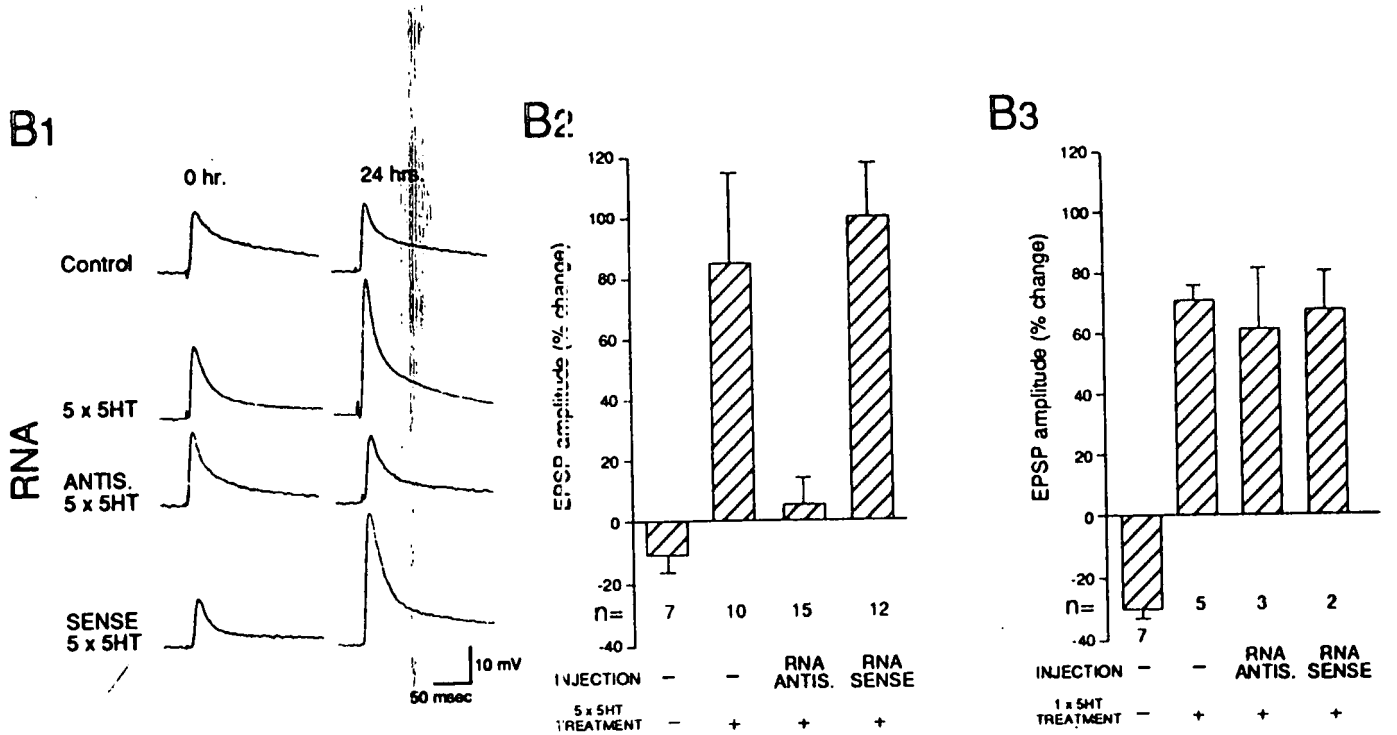


FIGURE 5B. Injection of ApC/EBP antisense RNA blocks 5-HT–induced long- but not short-term facilitation in the sensory motor synapses. **(B1)** Examples of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr) and 24 hr after the 5-HT treatment. Injection of the ApC/EBP antisense RNA but not of the ApC/EBP sense RNA prevents the 5-HT–induced increase in EPSP amplitude at 24 hr. The control culture did not receive 5-HT applications or RNA injections. **(B2)** *Bar graph* representing the effects of RNA injections in long-term facilitation. The height of each bar corresponds to the mean percentage change ±SEM in EPSP amplitude tested 24 hr after 5-HT treatment. A one-way analysis of variance and Newman Keuls' multiple-range test indicate that 5-HT treatment significantly increases the EPSP amplitude in noninjected cells as well as in ApC/EBP sense-RNA–injected cells relative to the control (not 5-HT–treated and noninjected cells) ($p < 0.01$). On the contrary, the EPSP amplitude change in ApC/EBP antisense-RNA–injected and 5-HT–treated cells was not significantly different from the control, nontreated, noninjected cultures. **(B3)** *Bar graph* representing short-term facilitation of cells injected with ApC/EBP antisense or sense RNA. A one-way analysis of variance and a comparison of the means show a significant effect of 5-HT in increasing EPSP amplitude in noninjected, in ApC/EBP antisense, or in ApC/EBP-sense RNA–injected cells compared to the control cultures that were neither injected nor 5-HT–treated ($p < 0.01$).

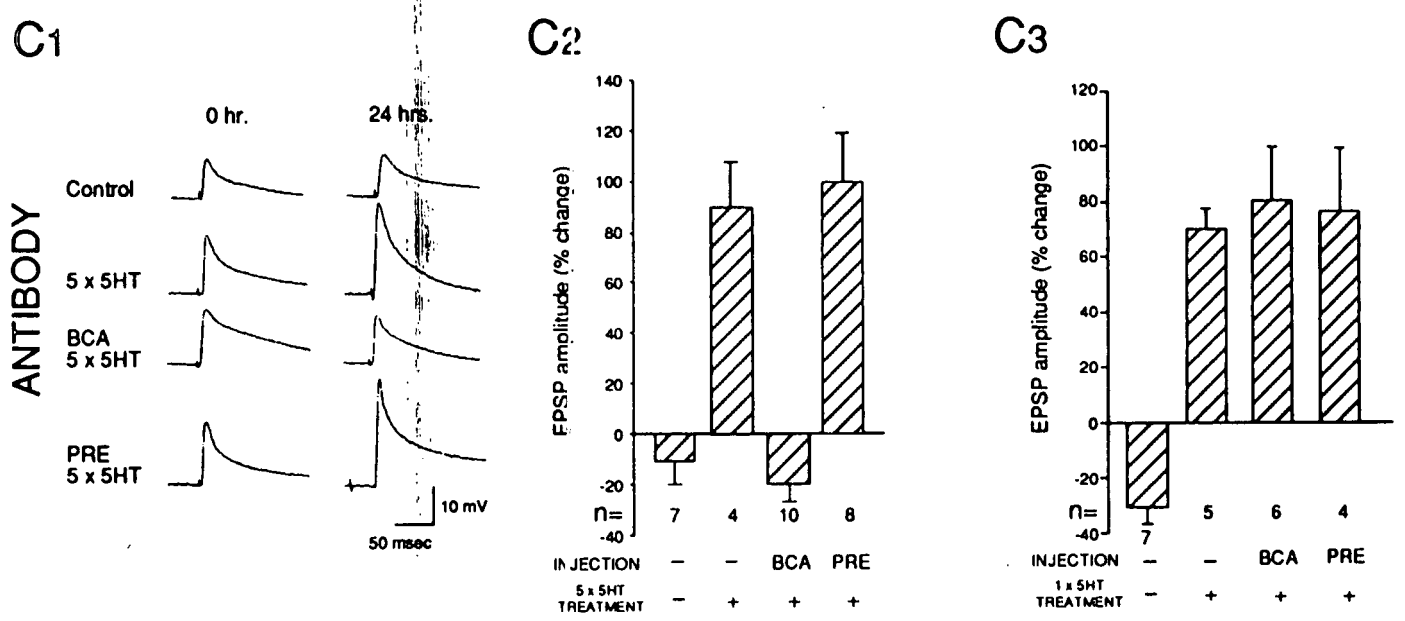


FIGURE 5C. Injection of antiserum anti-ApC/EBP (BCA) blocks 5-HT–induced long- but not short-term facilitation in the sensory motor synapses. **(C1)** Examples of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr) and 24 hr after 5-HT treatment. Injection of the antiserum BCA, but not of the preimmune serum (PRE) blocks the 5-HT–induced increase in EPSP amplitude at 24 hr. The control culture did not receive 5-HT applications or any injections. **(C2)** *Bar graph* representing the effects of injection of the antiserum BCA or the preimmune serum on long-term facilitation. The height of each bar corresponds to the mean percentage change ±SEM in EPSP amplitude tested 24 hr after 5-HT treatment. A one-way analysis of variance and Newman Keuls' multiple-range test indicate that 5-HT treatment significantly increases the EPSP amplitude in non-injected cells as well as in cells injected with preimmune serum relative to the control (not 5-HT–treated and noninjected cells) ($p < 0.01$). In contrast, the EPSP amplitude change in BCA injected cells was not significantly different from that observed in control cultures. **(C3)** *Bar graph* representing short-term facilitation of cells injected with BCA antiserum or preimmune serum. A one-way analysis of variance and a comparison of the means shows a significant effect of 5-HT in increasing EPSP amplitude in noninjected, in BCA-, or in PRE-injected cells compared to the controls that were neither injected nor 5-HT–treated ($p < 0.01$).

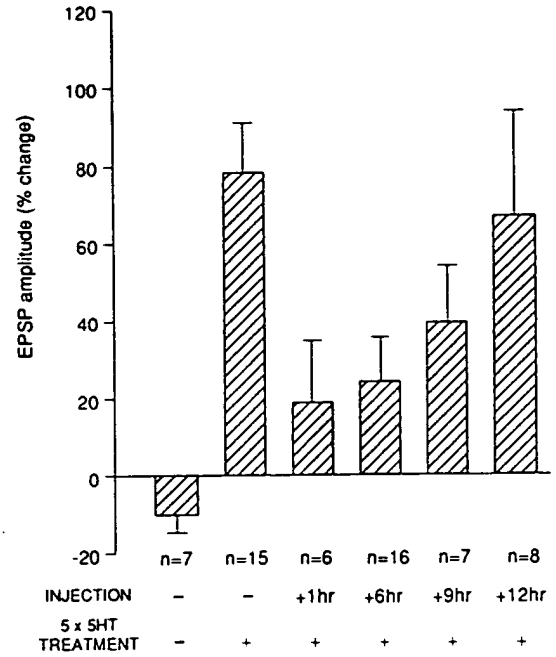


FIGURE 6. Time course of ERE effect following 5-HT treatment. *Bar graph* represents the percentage of change ±SEM in EPSP amplitude recorded 24 hr after five pulses of 5-HT from cocultures injected with ERE oligonucleotide at the indicated times after the end of 5-HT applications.

a cascade of self-perpetuating transcriptional events essential for the late phase of long-term facilitation.

cAMP-DEPENDENT GENE EXPRESSION IS A MOLECULAR SWITCH REQUIRED FOR CONSOLIDATION OF LONG-TERM FACILITATION IN *APLYSIA*

A schematic summary of the molecular events contributing to short- and long-term presynaptic facilitation is shown in FIGURE 7. The binding of serotonin to its surface receptors activates adenylyl cyclase, which catalyzes the synthesis of cAMP. cAMP binds to the regulatory subunit of the cAMP-dependent protein kinase (PKA), leading to the activation of its catalytic subunit. PKA acts on at least two classes of substrates to produce facilitation of the transmitter release. First, it phosphorylates K^+ channels or associated proteins, which leads to a reduction of the outward K^+ current and results in a broadening of the action potential and increased Ca^{2+} influx into the presynaptic neuron. Second, PKA also seems to act directly on the machinery in-

INJECTION – – BCA PRE
1 x 5HT TREATMENT – + + +

INJECTION – – BCA PRE
1 x 5HT TREATMENT – + + +

FIGURE 5C. Injection of antiserum anti-ApC/EBP (BCA) blocks 5-HT–induced long- but not short-term facilitation in the sensory motor synapses. (C1) Examples of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr) and 24 hr after 5-HT treatment. Injection of the antiserum BCA, but not of the preimmune serum (PRE) blocks the 5-HT–induced increase in EPSP amplitude at 24 hr. The control culture did not receive 5-HT applications or any injections. (C2) *Bar graph* representing the effects of injection of the antiserum BCA or the preimmune serum on long-term facilitation. The height of each bar corresponds to the mean percentage change ±SEM in EPSP amplitude tested 24 hr after 5-HT treatment. A one-way analysis of variance and Newman Keuls' multiple-range test indicate that 5-HT treatment significantly increases the EPSP amplitude in non-injected cells as well as in cells injected with preimmune serum relative to the control (nor 5-HT–treated and noninjected cells) ($p < 0.01$). In contrast, the EPSP amplitude change in BCA injected cells was not significantly different from that observed in control cultures. (C3) *Bar graph* representing short-term facilitation of cells injected with BCA antiserum or preimmune serum. A one-way analysis of variance and a comparison of the means shows a significant effect of 5-HT in increasing EPSP amplitude in noninjected, in BCA-, or in PRE-injected cells compared to the controls that were neither injected nor 5-HT–treated ($p < 0.01$).

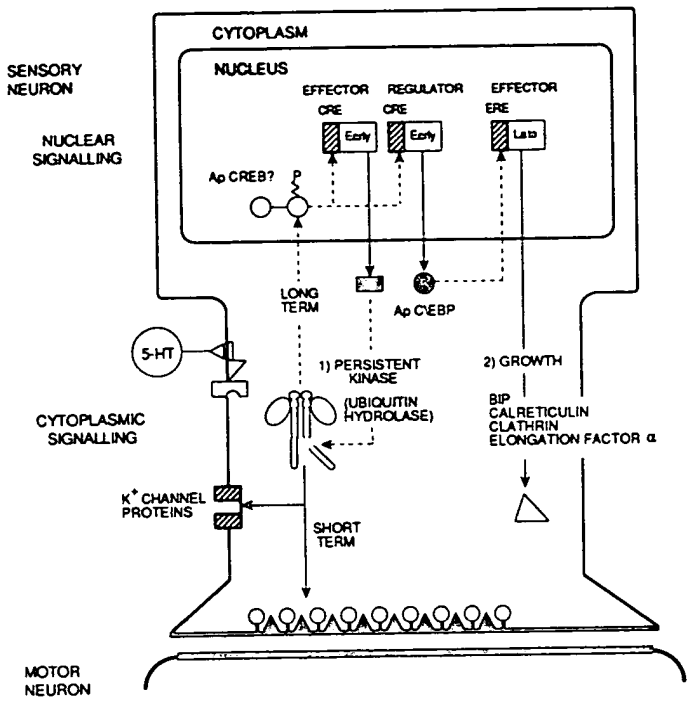
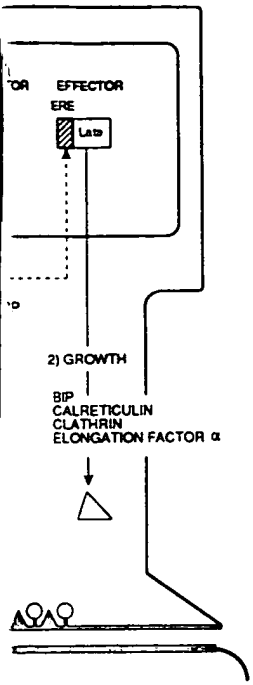


FIGURE 7. Schematic model of the activation pathways involved in *Aplysia* short- and long-term sensitization. See text for discussion.

volved in the exocytoric release of transmitter. These modifications take place in the presynaptic terminals and lead to short-term facilitation.

With repeated or prolonged application of 5-HT, the PKA catalytic subunit translocates to the nucleus, where it acts on nuclear substrates which appear to include transcription factors of the CREB/ATF family. Thus, the activity of CRE-binding proteins is necessary for long-term facilitation.

Transcription is the key event leading to long-term facilitation and the accompanying structural changes. This transcription-dependent phase includes the rapid induction of the transcription factor ApC/EBP, an immediate-early gene. This suggests that the induction of long-term facilitation requires the activation of a cascade of genes with constitutively active proteins regulating the expression of immediate-early genes, one of which is the transcription factor ApC/EBP. In turn, the early regulatory genes will likely lead to the expression of late target genes. The switching on of a self-maintaining mechanism by immediate-early genes explains why the characteristic protein synthesis-dependent phase is brief: the induction of regulatory factors is the limiting step that allows the expression of late phase events. In addition to regulatory factors, early effectors also are synthesized during the consolidation phase.



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Among these effectors is the C-terminal ubiquitin hydrolase that seems to participate in the proteolytic cleavage of the PKA regulatory subunit, maintaining the enzymatic activity of the catalytic subunit in the absence of a cAMP increase.[31,32]

Although PKA enzymatic activity is necessary for the first 10 hours following repeated 5-HT application, it is not maintained.[33] The late phase is characterized by morphological changes. Morphological studies have shown that structural changes appear within 1 hr after 5-HT or tail-shock training[34] and persist for days or weeks. Moreover, the decay of these structural changes seems to parallel the decay of behavioral memory.[35,36]

What molecular mechanisms underlie the formation of new synaptic connections? The synaptic growth is associated with a downregulation of NCAM-related apCAMs on the surface membrane of the sensory neuron.[37] Downregulation is achieved by activation of the endosomal pathway leading to internalization and apparent degradation of apCAM.[38] *Aplysia* expresses two types of isoforms, a transmembrane form and a phosphoinositol-linked form. Which of the two types of apCAM isoforms is internalized? To address this question, Craig Bailey, Bong-Kiun Kaang, and their colleagues selectively expressed epitope-tagged constructs of the two isoforms in cultured sensory neurons. By combining thin-section electron microscopy with gold-conjugated antibodies they have found that serotonin elicits a 68% decrease in the density of gold-labeled complexes bound to the transmembrane form of apCAM at the surface membrane, and a 24-fold increase in their internalization. By contrast, serotonin has no effect on either the surface distribution or internalization of the phosphatidylinositol-linked isoform of apCAM. The selective internalization of the transmembrane form highlights the potential regulatory significance of its intracellular domain, which contains a PEST sequence (thought to mediate protein degradation) and has two consensus sites for MAP kinase phosphorylation. Deletions of, or mutations in, the cytoplasmic tail should allow determination of which part of this molecule triggers internalization and which part targets degradation.

IMPLICIT FORMS OF LEARNING IN *DROSOPHILA* ALSO USE CREB AND THE cAMP CASCADE

Drosophila show classical conditioning to olfactory cues paired with shock. Several single gene mutants have been isolated that cannot learn the task although their behavior is otherwise normal. Two mutations have been analyzed in particular detail and each involves a step in the cAMP cascade.[39] *Dunce* involves a defect in the cAMP phosphodiesterase, whereas *rutabaga* is defective in the calcium-calmodulin–dependent adenylyl cyclase. Expression of an inhibitor of PKA using a heat-shock promoter also blocks the learning.

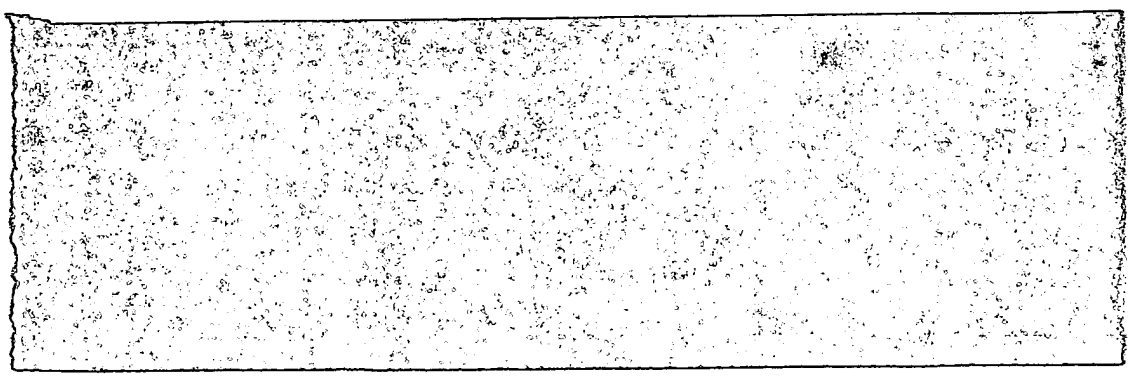
Recently, Tully *et al.*[13] have shown that spaced training gives rise to a long-term memory that lasts at least seven days and is blocked by inhibitors of protein synthesis. This long-term memory is selectively blocked by the heat-shock-induced expression of a dominant negative inhibitor of CREB, a CREM-like transcription repressor. Thus, several forms of long-term memory for implicit forms of learning require CREB- and cAMP-induced gene expression.

IMPLICIT AND EXPLICIT FORMS OF LEARNING SHARE SOME COMMON MECHANISMS

The studies in *Aplysia* suggest that the switch from short-term to long-term memory for simple reflexive forms of learning involves the induction by cAMP and CREB of a set of immediate early genes that participate in the growth of new synaptic connections that underlie the long-term process. Is there a similar molecular switch for memory consolidation in the mammalian brain that might contribute to the establishment of long-term memory storage for more complex explicit forms of learning?

Studies in humans and experimental animals have indicated that structures within the temporal lobe, such as the hippocampus, are particularly critical for explicit memory storage.[4] Are there cellular mechanisms within the hippocampus for storing explicit forms of memory? In 1973 Timothy Bliss and Terry Lømo first demonstrated that neurons in the hippocampus have remarkable plastic capabilities of the kind that would be required for learning.[40] A brief, high-frequency train of action potentials in any one of three neural pathways within the hippocampus produces an increase in synaptic strength in that pathway that can last for hours or days. This strengthening is called *long-term potentiation* or LTP.

LTP in the mammalian hippocampus shares some of the mechanisms used for synaptic facilitation in *Aplysia*. One form of LTP, called mossy fiber LTP, occurs at synapses between the dentate gyrus granule cells and CA3 pyramidal cells, and primarily involves a cAMP-dependent enhancement of transmitter release from the presynaptic terminals (FIG. 8). By contrast, Schaffer collateral LTP in CA1 is much more complex. Its induction involves calcium influx into the postsynaptic cell through the NMDA receptor channel and the recruitment in the postsynaptic cell of several second-messenger pathways involving tyrosine kinases, protein kinase C, and calcium/calmodulin kinase II. In addition to these inductive steps in the postsynaptic cell, Schaffer collateral LTP also involves an enhancement of transmitter release from the presynaptic neuron.[41–43] This enhanced release is thought to be mediated by one or more retrograde messenger signals (perhaps nitric oxide or carbon monoxide) that diffuse from the postsynaptic cell to the terminals of the presynaptic neuron.[44–46]



Similar to the presynaptic facilitation in *Aplysia*, both mossy fiber and Schaffer collateral LTP have distinct temporal phases, indicating that both short- and long-term memory have a cellular representation. There is an early phase produced by a single tetanic stimulation that lasts 1–3 hours and requires only covalent modification of preexisting proteins. In addition there is a late phase induced by repeated tetanic stimulation that persists for many hours, and is dependent on protein and RNA synthesis, and on PKA activity (FIG. 8).[47–49] As is the case with behavioral memory and presynaptic facilitation in *Aplysia*, there is a consolidation switch on the cellular level, and this requirement for transcription in LTP also has a critical time window.[50] Since the late transcription-dependent phase of LTP is blocked by inhibitors of PKA,[47,48] this suggests that in mammalian LTP as in *Aplysia* presynaptic facilitation cAMP-inducible genes need to be expressed (FIG. 8). Consistent with these findings, Bourtchuladze *et al.*[51] have found that mice that have a knockout of several critical CREB isoforms have a defect in LTP in the CA1 region that affects the late phase of LTP preferentially. These mice have normal learning and short-term memory of context conditioning, a hippocampal-based learning task, but they selectively lack long-term memory.

AN OVERALL VIEW

One of the key unifying findings emerging from these molecular studies is that the genetic switch for hippocampal LTP, required for explicit forms of learning, seems to share important similarities with that utilized in *Aplysia* and in *Drosophila*. Thus, molecular studies of cognition are revealing, on a mechanistic level, previously unsuspected similarities between different classes of learning, and suggest the interesting possibility that a common set of genetic mechanisms may be involved in a variety of learning-related long-term enhancements of synaptic transmission.

The data in *Aplysia* imply that these mechanisms include activation of immediate-early genes. Analysis of immediate-early gene expression in vertebrate brains has shown that many immediate-early genes are strongly induced in hippocampus and certain regions of the neocortex by treatments that lead to long-term potentiation.[52–55] It is now clear that CREB participates in hippocampal-based plasticity and long-term memory for explicit tasks. It will be of further interest to investigate whether cAMP-dependent immediate-early genes, perhaps of the C/EBP family, are also required for long-term neuronal plasticity in mammals.

The apparent conservation of some steps in the molecular mechanisms for long-term synaptic plasticity may reflect the fact that long-term memory storage commonly involves structural changes. Thus, in *Aplysia* the self-sustaining long-term process is expressed in the growth of new connec-

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